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Published in:
Cellular and molecular life sciences

DOI:
10.1007/s00018-004-4067-2

Publication date:
2004

CMLS, Cell. Mol. Life Sci. 61 (2004) 1185–1197
1420-682X/04/101185-13
DOI 10.1007/s00018-004-4067-2

CMLS Cellular and Molecular Life Sciences

Research Article

An extensive microarray analysis of AAL-toxin-induced cell death in *Arabidopsis thaliana* brings new insights into the complexity of programmed cell death in plants

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Received 12 February 2004; received after revision 5 March 2004; accepted 8 March 2004

Abstract. A T-DNA knockout of the *Arabidopsis* homologue of the tomato disease resistance gene Asc was obtained. The asc gene renders plants sensitive to programmed cell death (PCD) triggered by the fungal AAL toxin. To obtain more insights into the nature of AAL-toxin-induced cell death and to identify genes of potential importance for PCD, we carried out transcription profiling of AAL-toxin-induced cell death in this knockout with an oligonucleotide array representing 21,500 *Arabidopsis* genes. Genes responsive to reactive oxygen species (ROS) and ethylene were among the earliest to be upregulated, suggesting that an oxidative burst and production of ethylene played a role in the activation of the cell death. This notion was corroborated by induction of several genes encoding ROS-generating proteins, including a respiratory burst oxidase and germin oxalate oxidase. Cytochemical studies confirmed the oxidative burst and, in addition, showed synthesis of callose, a feature of the hypersensitive response. A diverse group of transcription factors was also induced. These events were followed by repression of most of the auxin-regulated genes known to be involved in growth and developmental responses. All photosynthesis-related genes were repressed. Blocking the synthesis of ethylene or NO significantly compromised cell death. In addition, we identified a heterogeneous group of early-induced genes, some of them never before associated with PCD. The group of early-induced genes included a number of proteases that were previously implicated in developmentally regulated types of PCD, suggesting a more principal role for these proteases in the PCD process. These findings provide new insights into the molecular mechanisms of plant PCD.

Key words. AAL toxin; PCD; hydrogen peroxide; transcriptome analysis.

Programmed cell death (PCD) is an active suicidal process that removes unwanted or severely damaged cells. PCD is an essential part of many developmental processes or responses to plant pathogens [1, 2]. While the signalling events and cell death cascades are well studied in animals, relatively little is known about the regulation and execution of PCD in plants. In particular, plant homologues of the key animal apoptotic proteases, caspases and the apoptotic regulators IAP and Bcl2 family proteins have not been found and there are no proteases with principal roles in the cell death process.

One of the well-characterized inducers of PCD in plants is the fungal AAL toxin [3]. PCD caused by the AAL toxin is an active process requiring de novo protein synthesis and exhibits all the hallmarks of apoptosis, including nuclear condensation and fragmentation. AAL toxin inhibits ceramide synthase, resulting in depletion of complex ceramides and accumulation of free sphingoid bases [4]. The tolerance to AAL-toxin-induced PCD depends, at least in tomato, on the presence of a gene involved in

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sphingolipid biosynthesis, called Asc [5], as Asc can to a large extent prevent the disruptions in sphingolipid metabolism and the subsequent cell death [6]. To use the power of *Arabidopsis thaliana* genomics, our group has identified a knockout of the *A. thaliana* homologue of Asc that rendered the mutant plants sensitive to growth inhibition by the AAL toxin [J. E. Markham and J. Hille, unpublished results]. Here we demonstrate that in the presence of AAL toxin, the knockout of Asc in *A. thaliana* leads to PCD with features resembling the hypersensitive response. To understand more about the nature of AAL-toxin-induced cell death and to identify genes and pathways that are differentially regulated during the process and that may have a role in PCD, we carried out a transcriptional analysis with an oligonucleotide array representing 21,500 *Arabidopsis* genes. Using such a wide genome coverage allowed us to identify a group of early-regulated genes, including transcription factors and proteases, that are potentially involved in the transcriptional reprogramming observed at the later stages and in the regulation of the PCD process. Some of these early genes have not yet been associated with PCD and therefore bring new insights into the nature of PCD. A number of ethylene and reactive oxygen species (ROS)-inducible genes were also upregulated very early. The molecular data were supported by cytochemical studies showing H_2O_2 accumulation and functional studies demonstrating that *de novo* synthesis of ethylene and NO are required for the enhancement of cell death.

Materials and methods

Plant material, growth conditions and toxin treatments

A. thaliana ecotype Wassilewskija LOH2 and wild-type (WT) plants were germinated and grown on soil under the following conditions: 14 h light/10 h dark period, photosynthetic photon flux density 100 µmol m^{-2} s^{-1}, 22°C and relative humidity 70%. Four-week-old plants were used for the experiments. AAL toxin (200 nM) was infiltrated in the leaves using a syringe without a needle as described previously [7]. Water-infiltrated plants served as controls. Samples were taken 7, 24, 48 and 72 h after the treatment and used for RNA isolation, conductivity measurements, visualization of the nuclear morphology and H_2O_2 determination.

Inhibition of ethylene production and NO synthesis

To inhibit ethylene production, AAL toxin was simultaneously infiltrated with 0.1 mg l^{-1} aminoethoxyvinylglycine (AVG), an ACC synthase inhibitor. NO synthesis was blocked with 5 mM N-methyl-L-arginine (L-NMMA), a nitrogen oxide synthase (NOS) inhibitor [8].

Measurements of cell death

Cell death was determined as electrolyte leakage from the leaves (increased conductivity). After sampling, plant leaves were immersed in ultrapure water for 30 min and the conductivity of the solution was measured with an LF-91 conductivity meter. Then the samples were briefly autoclaved in the same solution and the conductivity measured again (total conductivity). The increase in electrolyte leakage (conductivity) is expressed as percent of the total.

RNA isolation, microarray experiments and Northern analyses

For the microarray experiments, RNA was isolated with an RNAeasy Plant Mini Kit (Qiagen) according to the instructions of the manufacturer and treated with DNAse free of RNAse (Promega). RNA for the Northerns was isolated with the guanidine isothiocyanate method using TriReagent (Sigma). *A. thaliana* LOH2 plants were used both for the microarrays and the Northerns. The plants were pooled in two groups, one infiltrated with 200 nM AAL toxin and the other with water as control. Microarray experiments were performed in compliance with the MIAME standards [9]. We used the Arabidopsis2 oligonucleotide array of Agilent Technologies, representing 21,500 genes. RNA was isolated from AAL-toxin-treated samples and from H_2O-treated controls in order to exclude any side effects that might occur from the infiltration. Total RNA (20 µg) was used for the direct non-radioactive labelling procedure. The quality and quantity of the RNA was assessed with an Agilent Bioanalyzer. The labelling, hybridization and data extraction were done at ServiceXS (The Netherlands) according to the instructions of Agilent Technologies. Northerns were performed with 6 µg of total RNA as described elsewhere [6]. The quality and quantity of the RNA was verified by staining the membranes with methylene blue. For generation of radioactively labelled [^{32}P]dCTP probes, we used the following DNA stocks from TAIR: U09186 (TRX); 223N10 (GLP9); U09017 (Chl a/b BP); U13302 (GST); U14040 (GPx) and U17040 (WRKY). The PBB1 gene fragment was amplified from cDNA with primers PrRuG619 (TTTCAAGGATGGTGTGATACTAGGG) and PrRuG620 (CACCAACTTCTGTGATTTCGACTC).

Cytochemical techniques and microscopy

Nuclear morphology was visualized with 4′,6-diamidino-2-phenylindole (DAPI) staining. Briefly, leaves were fixed in ethanol:acetic acid (3:1, v/v) for 24 h, rinsed three times with water and stained with 0.1 mg ml^{-1} DAPI for 30 min. Leaves were then mounted on microscopic slides with glycerol and examined under UV light with a Carl Zeiss fluorescent microscope, DAPI filter. Hydrogen peroxide production was determined by cytochemi-

cally staining leaves with 3,3′-diaminobenzidine (DAB). DAB forms a brown precipitate when oxidized by H_2O_2. Leaves were infiltrated with 5 mM DAB 30 min before sampling. After sampling, leaves were fixed in ethanol for 1 h and examined for DAB deposits under a microscope. For the detection of callose, leaves were fixed in 70% ethanol, cleared in chloral hydrate, rinsed three times in water and stained overnight in 0.01% aniline blue. The blue fluorescence was visualized under UV light with a Carl Zeiss fluorescent microscope, DAPI filter.

Results

AAL toxin causes cell death in Asc knockout *A. thaliana*

The AAL toxin causes disruption in sphingolipid metabolism and subsequent PCD in tomato [3]. Although the exact mechanisms of cell death activation and execution remain elusive, the presence of a disease resistance gene called Asc is known to prevent the perturbations in sphingolipid metabolism and cell death [6]. Asc is homologous to the yeast longevity assurance gene LAG1 [5]. Our group established that a knockout of the Asc homologue in *A. thaliana* rendered plants significantly more sensitive to AAL toxin. The *A. thaliana* knockout was called LOH2 (LAG one homologue 2). The LOH2 plants exhibited necrotic lesions 3 days after the leaves had been infiltrated with 200 nM AAL toxin (fig. 1B) and the cell death was also demonstrated by increased electrolyte leakage (fig. 1C). The cell death was light dependent, as there was no death in the dark (data not shown). No necrotic spots or increased electrolyte leakage were observed 7 and 24 h after the toxin infiltration and only a small increase in electrolyte leakage was recorded after 2 days. The cell death in LOH2 was preceded by nuclear condensation, as indicated by fluorescent labelling with DAPI (fig. 1A). Nuclear condensation is a feature of PCD in both animals and plants [10]. In contrast, the leaves from wild-type plants remained necrosis free, no nuclear condensation was observed (data not shown) and no increase in electrolyte leakage was recorded throughout the experiment (fig. 1C).

Transcriptional analysis of AAL-toxin-induced cell death

Despite linkage between the perturbations in sphingolipid metabolism and PCD, little is known as to how the

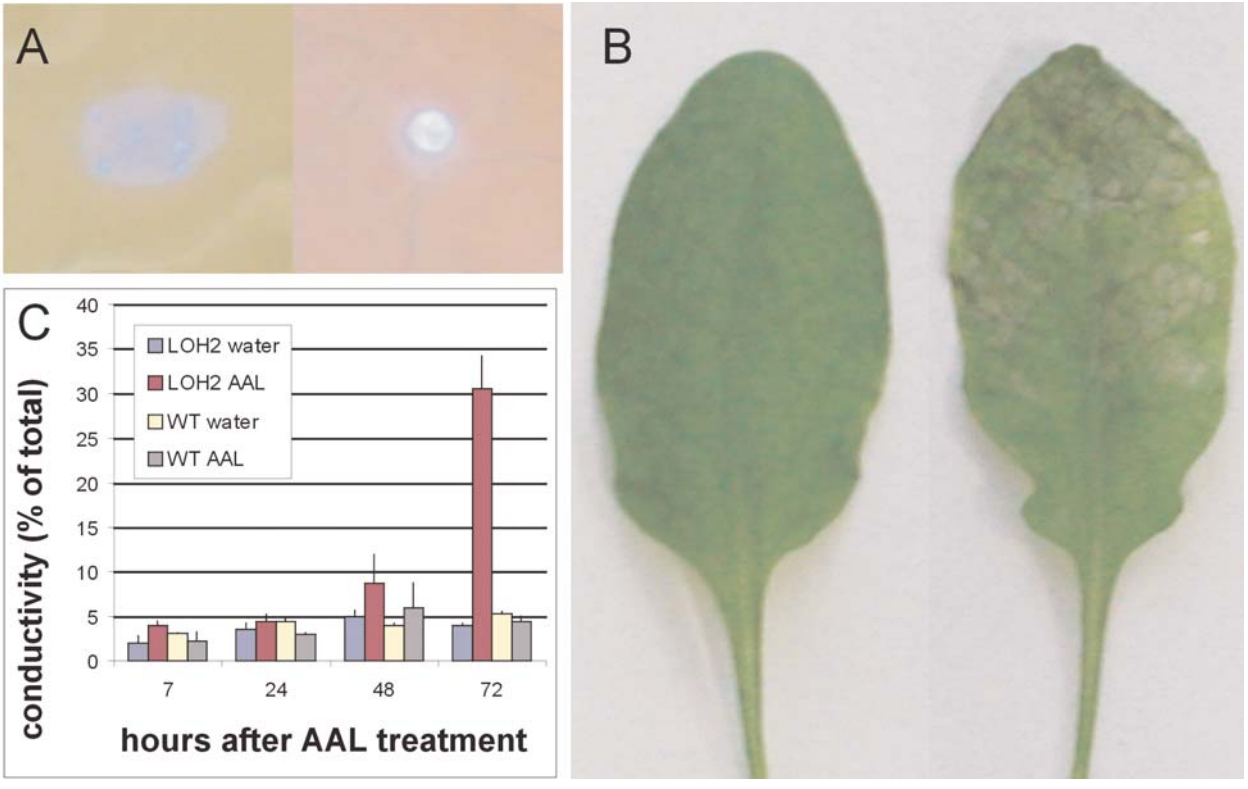

Figure 1. AAL toxin triggers cell death in plants with perturbed sphingolipid metabolism (LOH2 plants). Leaves of LOH2 and WT plants were infiltrated with 200 nM AAL toxin or water (controls). (A) Nuclear condensation, DAPI staining. Left, water-treated LOH2. Right, AAL-toxin-treated LOH2, 3 days after AAL toxin application. (B) Leaf necrosis. Left, water-treated LOH2. Right, AAL-toxin-treated LOH2, 3 days after application of AAL toxin. (C) Electrolyte leakage. Samples were taken at 7, 24, 48 and 72 h and electrolyte leakage assessed as increased conductivity. Data are means ± SD, n = 3.

cell death is actually triggered and about which genes are involved in the regulation or execution of the process. To obtain more information about the nature of the AAL-toxin-triggered cell death and to identify genes and pathways that are differentially regulated, we carried out a four-time-point transcriptional analysis with an oligonucleotide array representing 21,500 genes from *A. thaliana*.

Samples of AAL-toxin-treated leaves were taken 7, 24, 48 and 72 h after the treatment. At the first time point, 81 genes were regulated more than 3.5 times. That number increased to 199 genes at 24 h and 2288 genes at 48 h, demonstrating that a massive transcriptional reprogramming took place between the first and the second day of the AAL toxin treatment.

Table 1 presents the most regulated genes at 7 and 24 h as well as marker genes for different pathways including hormone signalling and perception, sphingolipid metabolism, photosynthesis and protein degradation. The full dataset is available at our website at http://www.rug.nl/biologie/onderzoek/onderzoekgroepen/MolecularBiologyofPlants/onderzoek/copyofSupplementalTable1_AAL.xls

To verify the microarray data, we performed Northern analyses with genes selected from the microarrays. The selected genes included ROS- and stress-responsive GST, GPx and TRX, the germin oxalate oxidase GLP9, a chlorophyll a/b-binding protein, the proteasome subunit PBB1, and a WRKY transcription factor (fig. 2). Quantification of the signals showed that all Northerns

Table 1. Global changes in gene expression during AAL-toxin-induced cell death.

Function	Gene locus	Description	Fold change after the AAL treatment			
			7 h	24 h	48 h	72 h
Transcription factors	AT1G57560.1	myb family transcription factor	2.1	3.2	12	19
	AT5G18270.1	NAM (no apical meristem)-like protein	1.9	4.8	12.9	12
	AT2G43000.1	NAM (no apical meristem)-like protein	1	8.1	25.2	23.4
	AT2G38340.1	DREB-like AP2 domain transcription factor	1	4.4	22	33.8
	AT5G43650.1	bHLH protein	3.2	4.3	3.1	2.9
	AT3G53600.1	zinc finger-like protein	3	3.2	5	4
	AT2G37430.1	putative C2H2-type zinc finger protein	4.5	3	4	6.7
	AT1G74430.1	myb family transcription factor	3.4	1	–4	–4.6
	AT4G36900.1	AP2 domain protein RAP2.10	2.7	–1.5	–5.8	–8.3
	AT1G68520.1	CONSTANS B-box zinc finger family protein	1	–2.2	–12.6	–11
	AT5G24930.1	CONSTANS B-box zinc finger family protein	–2.5	–1.9	–10	–17.5
	AT4G25470.1	DRE-binding protein (DREB1C)	–3.9	1	–5	–7
Ethylene responses	AT5G43410.1	*Nicotiana* EREBP-3-like	5.8	2.8	5.4	5.2
	AT1G01480.1	ACC synthase	1.3	1	5	8.7
Antioxidant metabolism and oxidative burst	AT5G51060.1	respiratory burst oxidase protein	3.5	2.4	18	46
	AT1G14540.1	anionic peroxidase, putative	4.6	5	18.6	19.5
	AT5G19880.1	peroxidase, putative	1.4	8	37	38
	AT4G14630.1	germin precursor oxalate oxidase	1	13	56	46.5
	AT5G38910.1	germin-like protein	1	4	30.8	100
	AT5G38960.1	germin-like protein	1	4.7	75.5	17.8
	AT1G45145.1	thioredoxin, putative	2	1.6	11.5	12
	AT1G65970.1	type 2 peroxiredoxin, putative	1.3	2.6	35.5	75.7
	AT2G31570.1	glutathione peroxidase, putative	1.3	2.5	15.8	14
	AT2G29470.1	glutathione transferase, putative	2.8	7.1	81	100
	AT1G69930.1	glutathione transferase, putative	4.8	4	14	11.7
	AT2G29480.1	glutathione transferase, putative	2.4	3	51	87
Auxin signalling and responses	AT5G18010.1	auxin-induced protein (SAUR), putative	1.9	1	–39	–22.6
	AT1G15580.1	auxin-induced protein IAA5, putative	1.9	–1.7	–7.2	–7.9
	AT5G18020.1	auxin-induced protein-like	1.6	1	–52	–22.3
Jasmonic acid signalling	AT2G39940.1	coronatine-insensitive 1 (COI1), AtFBL2	1	1	1.3	1
	AT1G72260.1	thionin	1	1	1	1
	AT5G36910.1	thionin Thi2.2	1.7	1	1	1
	AT5G44020.1	vegetative storage protein-like	1	–1.5	–5	–4.5
Gibberellic acid signalling	AT1G22690.1	putative gibberellin-regulated protein	1.3	1.3	3	4
	AT1G30040.1	gibberellin 2-oxidase (GA2-oxidase) (ga2ox2)	2.2	1.1	2.7	5
	AT2G34555.1	gibberellin 2-oxidase (GA2-oxidase) (ga2ox3)	1.8	1	2.5	2.5
Abscisic acid/ abiotic stress	AT4G24960.1	abscisic acid-induced-like protein	1.2	–3.6	–3	–1.9
	AT5G52310.1	low-temperature-induced protein 78 (sp|Q06738)	–4.7	–5	–8.1	–4.4
	AT5G52300.1	low-temperature-induced 65-kDa protein	–5	–1.6	–5.5	–3.5
	AT2G42540.1	cold-regulated protein cor15a precursor	1	–3.2	–20	–12
	AT3G50980.1	dehydrin-like protein	–2.2	–3.8	–15	–11

Table 1 (continued)

Function	Gene locus	Description	Fold change after the AAL treatment			
			7 h	24 h	48 h	72 h
Biotic stress/defence	AT1G74200.1	putative disease resistance protein	–1.3	–4.5	–20	–14.5
	AT1G72890.1	disease resistance protein (TIR-NBS class), putative	1.9	3.8	20	22
	AT5G66890.1	disease resistance protein (NBS-LRR class), putative	1	5.1	11.5	8.4
	AT2G35980.1	similar to harpin-induced protein hin1 from tobacco	1.7	10	66	100
	AT2G26020.1	plant defensin protein, putative (PDF1.2b)	1	2.1	5	13
	AT5G13080.1	WRKY family transcription factor	3.8	7.2	68	100
	AT4G22070.1	WRKY family transcription factor	1.3	8.9	32	100
	AT5G64810.1	WRKY family transcription factor	1.3	4	13	10
	AT5G22570.1	WRKY family transcription factor	1	4	8.8	4.8
	AT3G19690.1	PR–1 protein, putative	1	2.7	6.1	7.7
	AT1G75040.1	thaumatin-like protein	1	10	18.6	56
	AT3G04720.1	hevein-like protein precursor (PR-4)	1	1.8	12.3	27
	AT3G12500.1	glycosyl hydrolase family 19 (basic endochitinase)	1.7	3.7	32.4	67.6
	AT1G65610.1	glycosyl hydrolase family 9 (endo–1,4-beta-glucanase)	2.6	5.5	20	48
	AT3G03640.1	glycosyl hydrolase family 1	1	5	20.6	20.6
	AT1G61820.1	glycosyl hydrolase family 1	1	13.2	43	40
	AT5G36970.1	harpin-inducing protein (hin1), *Nicotiana tabacum*	1	3.9	36	23.8
	AT1G65690.1	expressed protein, similar to hin1	1	3.4	21.9	25
	AT1G61560.1	Mlo protein, putative	4	2.2	5.6	5.7
Photosynthesis	AT4G27440.1	protochlorophyllide reductase precursor	–2	–1.4	–25	–10
	AT5G38410.1	ribulose bisphosphate carboxylase small-chain 3b precursor (RuBisCO small subunit 3b) (sp|P10798)	1.6	1.3	–2.4	–2.2
	AT2G05070.1	light-harvesting chlorophyll a/b-binding protein	–2.2	–1.7	–25	–15
Housekeeping/ diverse	AT3G05640.1	protein phosphatase 2C (PP2C), putative	1	–3.6	–15.8	–5.5
	AT4G08260.1	protein phosphatase 2C (PP2C), putative	–3.8	1	–5.9	–3.3
	AT3G53750.1	actin (ACT3)	1	1	1	–1.6
	AT1G76650.1	EF-hand-containing protein	3	2.8	1	1
	AT1G15520.1	ABC transporter family protein	–1.3	10.6	79	100
	AT1G55780.1	heavy metal transport protein	1.8	11.6	30.6	39.8
	AT1G55780.1	hypothetical protein (heavy metal transport protein)	1.8	11.5	31.6	40
	AT5G17330.1	glutamate decarboxylase 1 (GAD 1)	1	4	13	13
	AT5G45180.1	dimethylaniline monooxygenase (NO-forming)-like protein	1	1.9	14.1	7.4
	AT1G19250.1	FMO (NO-forming)	1	10	47.9	100
	AT2G28210.1	putative carbonic anhydrase	1	10	53.7	70.8
	AT2G13810.1	putative aspartate aminotransferase	1.5	9.2	53.7	100
	AT1G61820.1	glycosyl hydrolase family 1	1	13.2	42.7	42
	AT3G13610.1	2-oxoglutarate-dependent dioxygenase family	2.8	10.5	53.7	100
	AT4G20800.1	FAD-linked oxidoreductase family	2.2	16.5	85	100
	AT1G26390.1	FAD-linked oxidoreductase family	1	8.5	31.5	100
	AT1G26400.1	FAD-linked oxidoreductase family	1.6	21	86	100
	AT1G26380.1	FAD-linked oxidoreductase family	2	6.3	57.5	98
	AT1G26420.1	FAD-linked oxidoreductase family	2.3	5.8	47.9	81.3
	AT3G26830.1	cytochrome p450 family	1.5	5.9	25	73.3
	AT1G11610.1	cytochrome P450, putative	1	3.9	18	26.3
	AT2G30770.1	cytochrome p450 family	1.8	4.5	21.5	100
	AT3G01420.1	feebly-like protein	1	5	45.8	79.4
	AT2G22470.1	arabinogalactan-protein (AGP2)	1	4.4	26.3	52.5
	AT1G35230.1	arabinogalactan-protein (AGP5)	1.6	3.7	17.3	27.5
	AT1G66700.1	methyltransferase-related	1	3.6	20	24
	AT5G48400.1	ligand-gated ion channel protein-like; glutamate receptor-like	1	4	63	60
	AT3G48850.1	mitochondrial phosphate transporter	1.7	5.4	32.4	32.3
	AT3G49620.1	oxidoreductase (din11), putative	1	3.9	66	8.5
	AT1G09935.1	similar to ZW10-like protein	1	4.5	52.5	10
	AT5G48410.1	ligand-gated ion channel protein-like; glutamate receptor-like	1.9	4.3	37.2	100
	AT5G02780.1	In2-1 protein, putative	1	5.6	55.5	72.4
	AT2G14620.1	xyloglucan endotransglycosylase, putative	1	6.9	60	100
	AT5G38900.1	frnE protein-like	1	5.1	33.5	79.5
	AT1G61800.1	glucose-6-phosphate/phosphate-translocator precursor, putative	1.7	4.3	37.2	89
	AT1G68620.1	similar to PrMC3 (*Pinus radiata*)	1.7	5.2	39.8	50

Table 1 (continued)

Function	Gene locus	Description	Fold change after the AAL treatment			
			7 h	24 h	48 h	72 h
	AT5G46950.1	similar to pistil-specific gene sts15 (*Solanum tuberosum*)	1	9	29	50
	AT5G44480.1	NAD-dependent epimerase/dehydratase family, similar to SP|P55180 UDP-glucose 4-epimerase	1	3.7	20	40
	AT4G26260.1	putative protein PRE87 mRNA, *Pinus radiata*	1	3	20.9	58.9
	AT1G36370.1	putative hydroxymethyltransferase	3.3	1.5	–3	–8.8
	AT5G06870.1	polygalacturonase-inhibiting protein (PGIP2)	2.8	1	–7.6	–8.5
Ubiquitin-proteasome pathway	AT3G27430.1	20S proteasome beta subunit B (PBB1)	1	1	2	2.9
	AT3G20630.1	ubiquitin-specific protease 14 (UBP14), putative	1	2	4.5	4.4
	AT5G20000.1	26S proteasome AAA-ATPase subunit RPT6a	1.4	1.5	6	6.3
	AT5G03240.1	polyubiquitin (UBQ3)	1	1.3	4.4	12.3
	AT4G09100.1	C3HC4-type zinc finger protein family	1	4.5	16.2	13.5
Proteases	AT1G32960.1	subtilisin-like serine protease	2.1	2.9	12.9	85
	AT1G44130.1	nucellin	1.7	5.3	18.2	23
	AT1G70170.1	matrix metalloproteinase, putative	2	4.2	14.5	21.7
	AT5G26860.1	Lon protease homolog 2 precursor	1	2.1	20.9	7.2
	AT1G32940.1	subtilisin-like serine protease	1.3	1.7	5.5	20
	AT5G45890.1	senescence-specific cysteine protease SAG12	1	1	1	1.5
	AT3G22600.1	protease inhibitor/seed storage/lipid transfer protein family	1.5	9.1	49	95
Sphingolipid metabolism	AT3G25540.1	LAG1	1	1	2.8	1.7
	AT1G13580.1	LAG3	1	1	1	1
	AT2G34690.1	Acd11	1	1	1	1
	AT3G48780.1	AtLCB1	1	1	2.5	3.2
	AT5G23670.1	AtLCB2	1	1	1	1
	AT4G36480.1	AtLCB3	1	1	1.6	1.7
	AT5G23450.1	AtLCBK1	1	1	1	1
	AT4G21540.1	AtLCBK2	1	1	2.4	2
	AT3G58490.1	AtLCBP1 phosphatidic acid phosphatase	1.6	1	1	1
	AT1G27980.1	AtDPL1	1	1	3	2.8
	AT5g51290.1	Acd5	1	1	2.6	2
Putative/expressed proteins	AT3G10020.1	expressed protein	–2.2	–3.7	–17	–8.2
	AT2G32190.1	expressed protein	3.9	3.6	20	48.6
	AT2G35730.1	expressed protein	2.6	3.6	31.6	91.2
	AT5G53870.1	contains similarity to phytocyanin/early nodulin-like protein	1	5	17.5	13.5
	AT5G62150.1	putative protein	1	4	33	37
	AT3G61390.1	putative protein	2.5	6.3	36.8	88.5
	AT5G56640.1	putative protein	1	4.6	100	13.8
	AT5G06230.1	putative protein	1	6.3	17.8	8.2
	AT2G36020.1	expressed protein	1	4.7	27	9
	AT1G03660.1	unknown	1	6.6	25	49
	AT1G56060.1	hypothetical protein	1	3.7	15.8	30.8

Leaves of LOH2 plants were infiltrated with water (controls) or 200 nM AAL toxin. Samples were collected 7, 24, 48 and 72 h after the AAL treatment. Genes represented in the table include the most regulated at the two early time points as well as marker genes for sphingolipid metabolism, hormone pathways, antioxidant metabolism, stress and defence, as well as photosynthesis-related genes, components from the proteasome pathway, and the most upregulated proteases.

were consistent with the microarray results (data not shown).

At 7 h, the most upregulated gene was EREBP-3-like (At5G43410, table 1). Other upregulated ethylene-response-related genes at that time included two EREBPs (AT2G44840 and AT5G61590, supplementary table 1) and an ethylene-induced calmodulin-binding protein (AT2G22300). Two glutathione-S-transferases (GSTs), known to be responsive to elevated H_2O_2 levels, were also upregulated at that time point (table 1, fig. 2) suggesting that accumulation of ROS was also an early event triggered by AAL toxin. In addition to EREBPs, other transcription factors upregulated that early were a putative C2H2-type zinc finger protein and two MYB family transcription factors (table 1). On the other side, a CONSTANS B-box zinc finger family protein was downregulated (table 1). Interestingly, several abiotic stress genes and a protein phosphatase 2C (AT4G08260.1) were downregulated as well.

At 24 h, the above-mentioned genes showed consistent trends of expression. However, many new genes appeared to be regulated at that point. Moreover, some of those

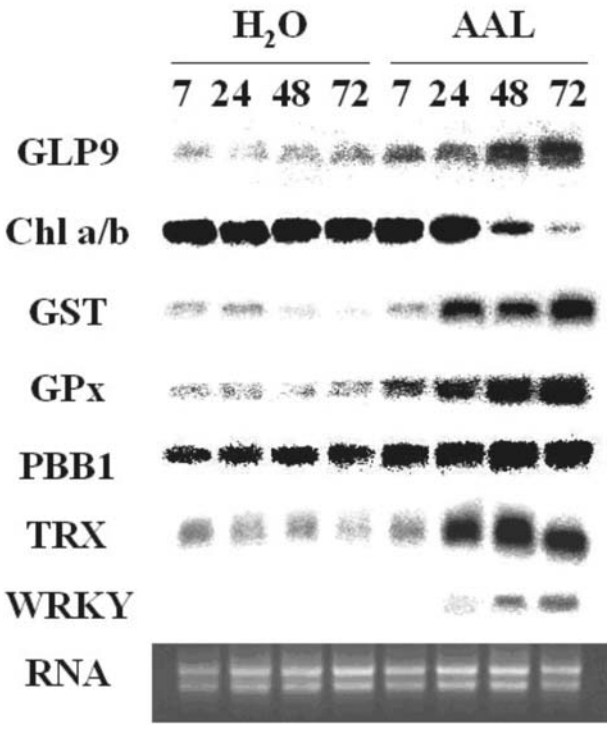

Figure 2. Expression profiling during AAL-toxin-induced cell death in LOH2 plants. The expression pattern of selected genes from the microarray experiments (table 1) was verified by Northern analysis. Leaves of LOH2 plants were infiltrated with either water (controls) or 200 nM AAL toxin and samples taken at 7, 24, 48 and 72 h. Genes are as follows: GLP9, germin oxalate oxidase (At4G14630.1); Chl a/b, light-harvesting chlorophyll-a/b-binding protein (At2G05070.1); GST, glutathione-S-transferase (At2G29470.1); GPx, glutathione peroxidase (At2G31570.1); PBB1, 20S proteasome β subunit (At3G27430.1); TRX, thioredoxin (At1G45145.1); WRKY (At5G13080.1).

genes were highly regulated with a more than 10-fold change, in contrast with the situation at 7 h where the most upregulated gene had a 5.8-fold change (table 1, supplementary table 1). Some of the newly upregulated genes at that point were transcription factors: several no apical meristem (NAM)-like and other WRKY family members. Consistent with the induction of WRKY, several pathogenesis-related (PR) genes were upregulated, including PR-1 (AT3G19690), PR-4 (AT3G04720), and PR-5 (AT1G75040) (table 1). Corroborating the occurrence of an oxidative burst was the strong induction of several ROS-generating enzymes. These included a respiratory burst oxidase (AT5G51060; table 1), germin-precursor oxalate oxidase GLP9 (AT4G14630; table 1, fig. 2), as well as several other peroxidases and oxidoreductases with the ability to generate ROS. GLP9, for example was among the most upregulated genes at 24 h with more than 13-fold induction. These data suggested a biphasic oxidative burst with a stronger second phase of sustained ROS production [11]. The most upregulated genes at 24 h also included members from the FAD-linked oxidoreductase family, several antioxidant enzymes, a number of putative disease resistance genes, glycosyl hydrolases, 2-oxoglutarate-dependent dioxygenases, ABC transporter family members, a heavy metal transport protein (At1G55780), putative carbonic anhydrase and aspartate aminotransferase, a flavine monooxygenase and various transcription factors (table 1). At the same time, a few other antioxidant enzymes and several transcription factors were repressed, demonstrating the complexity of the responses. At 24 h, we also observed induction of several different proteases: a subtilisin-like serine protease (At1G32960), an aspartatic protease (At1G44130), and a matrix metalloproteinase (At1G70170). These three proteases were barely detectable in the water-treated samples. The latter two are particularly interesting, because similar proteases were previously associated with nucellar cell degeneration and senescence, respectively, two types of developmentally controlled PCD [12, 13].

The genes regulated at 24 h not only retained their expression pattern at the next time points but further increased their degree of regulation. For example, the ABC transporter AT1G15520.1 showed 10.6-fold upregulation at 24 h, 79-fold at 48 h and more than 100-fold at 72 h. At 48 h, by which time a massive transcriptional reprogramming has taken place, we observed repression of the majority of auxin-regulated genes (table 1). All photosynthesis-related genes were also repressed. We also observed induction of several elements of the proteasome pathway, among them ubiquitin-specific protease UBP14 (At3g20630), a RING-finger protein (At5G55970), and genes from the regulatory and catalytic subunits of the proteasome (table 1, fig. 2). The ubiquitin-proteasome pathway is not merely involved in degradation of proteins during stress and cell death responses; rather, it plays a major role in regulating the PCD process and in animals it interplays with other components of the cell death machinery, most notably caspases [14].

In contrast to the ROS and ethylene, there was no indication for accumulation of the plant hormones jasmonic acid (JA), gibberellic acid (GA) and cytokinin. The thionin genes, arguably the best markers for the presence of JA [15], were hardly detectable and were not induced at all (table 1). Other JA markers like PINs and VIPs were even downregulated. Likewise, GA-regulated proteins were either not changed or downregulated, while several GA2 oxidases that would degrade GA [16] were actually induced in our samples. Most of the genes implicated in cytokinin responses were unchanged, while a cytokinin-like oxidase was moderately induced after 48 h and the cytokinin response regulator ARR6 (At5G62920) was also negatively regulated.

There were no significant changes in transcripts of genes involved in sphingolipid metabolism (table 1). If such genes are involved in the PCD process they are apparently not regulated at the level of transcription; rather, they may

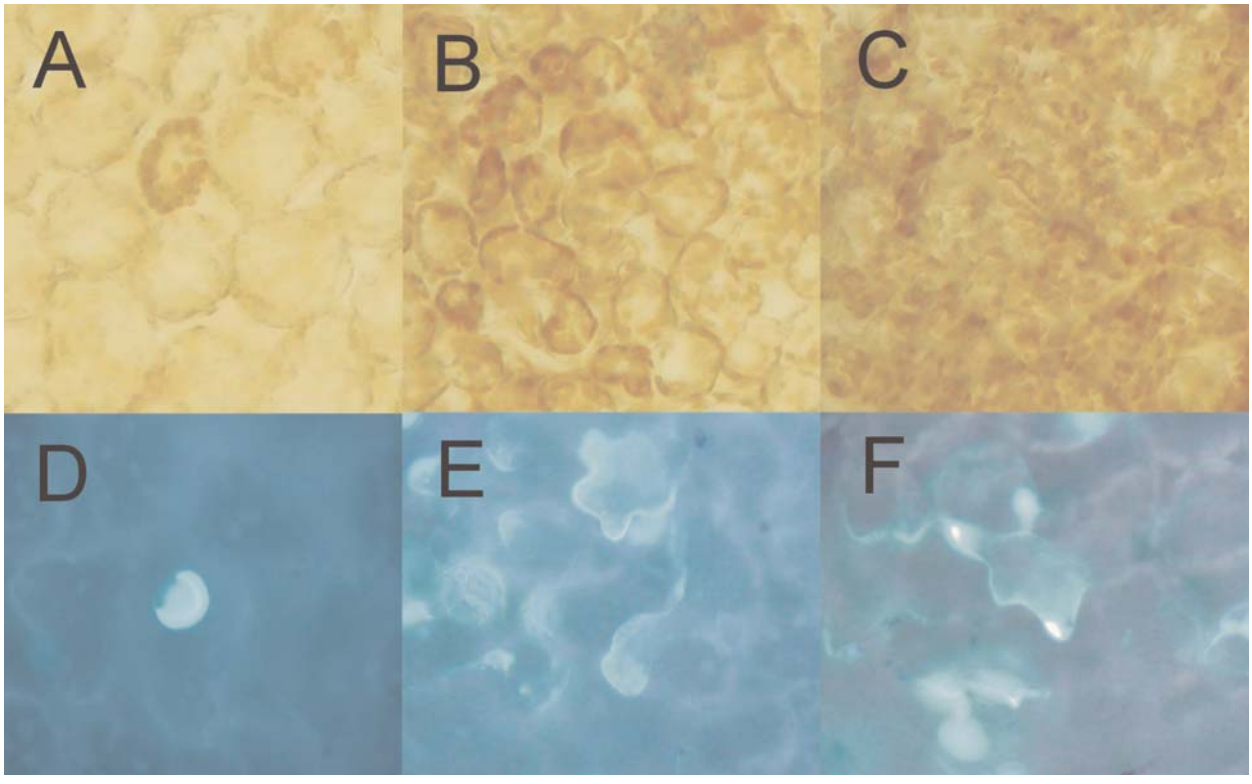

Figure 3. AAL toxin causes accumulation of H_2O_2 and synthesis of callose. Leaves of LOH2 plants were stained with DAB for H_2O_2 determination (A–C) and with aniline blue for detection of callose (D–F) as described in Materials and methods. DAB forms a brown precipitate when oxidized by H_2O_2 and callose fluoresces blue under UV light. For H_2O_2 determination pictures are from samples taken 7 (A), 24 (B), and 48 (C) h after the toxin treatment. For the callose, pictures are from samples taken 24 (D), 48 (E), and 72 (F) h after the toxin treatment. No callose was detected at 7 h.

be regulated posttranscriptionally. Such a scenario is easily envisaged as in the case, for example, with the recently cloned ceramide kinase which has to be activated rather than transcriptionally upregulated [17].

AAL toxin causes an oxidative burst and the cell death that follows is enhanced by ethylene and NO

Given the indications for an oxidative burst from the microarray data, we studied the accumulation of hydrogen peroxide by in situ staining for H_2O_2 with DAB. DAB is rapidly oxidized by H_2O_2 and forms brown precipitates at the sites of H_2O_2 production. Consistent with the microarray data, we noticed first signs of H_2O_2 production as early as 7 h, when single leaf cells were stained in brown (fig. 3A). However, much more significant staining of big clusters of cells was observed at 24 h (fig. 3B), which coincided with the switching on of several peroxidases, FAD-linked oxidoreductases and a germin oxalate oxidase. The staining for H_2O_2 was strong at 48 h (fig. 3C) and at 72 h (data not shown) as well. The oxidative burst at 24, 48 and 72 h was accompanied by synthesis of callose (fig. 3D–F).

To investigate the role of ethylene in the activation of the cell death process, LOH2 plants were infiltrated simultaneously with AAL toxin and AVG. AVG is an effective ethylene biosynthesis inhibitor and is much less toxic to plants than silver nitrate, another commonly used ethylene interference agent that blocks ethylene receptors [W. van Workum, personal communication]. The cell death was then quantified by measuring the electrolyte leakage and compared with the cell death caused by AAL toxin alone. The results showed that AVG was able to reduce the cell death by about 50% 3 days after the treatment (fig. 4).

NO is another signalling molecule that was shown to be essential for triggering PCD in plant-pathogen interactions [18]. To investigate a possible involvement of NO in our cell death system, we took advantage of the specific NO inhibitor L-NMMA. L-NMMA was also able to reduce the cell death by half (fig. 4), demonstrating that NO as well as ethylene are required for the enhancement of PCD.

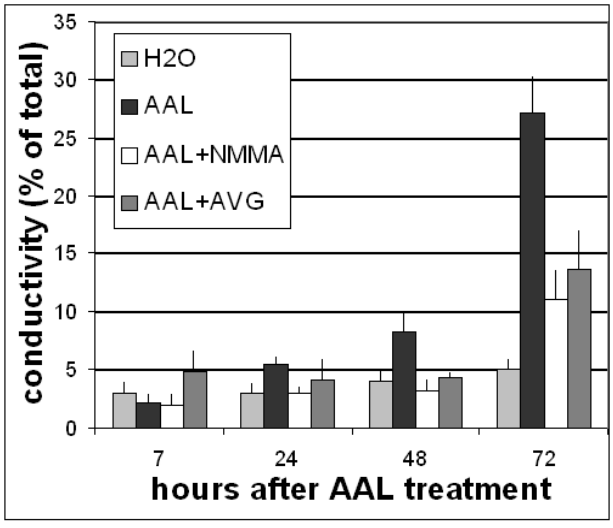

Figure 4. Inhibition of ethylene or nitric oxide synthesis decreases AAL-toxin-induced PCD. Leaves of LOH2 plants were infiltrated with water (controls) or 200 nM AAL toxin or a combination of AAL toxin + AVG, an ethylene synthesis inhibitor, or AAL toxin + L-NMMA, a NOS inhibitor, and cell death measured as increased conductivity at 7, 24, 48 and 72 h after the treatment. Data are means ± SD, n = 3.

Discussion

AAL-toxin-sensitive *Arabidopsis*: a powerful tool to study PCD

Cell death in AAL-toxin-sensitive LOH2 can be triggered by nanomolar concentrations of the toxin. Results after the AAL toxin treatment are very reproducible with respect to timing of gene expression, morphological effects and cell death. The AAL toxin triggers PCD through interference with sphingolipid metabolism, although the exact mechanism of activating the cell death machinery remains elusive [3, 6]. The role of sphingolipids in promoting cell proliferation or death is well recognized in animals [19]. Recently, sphingolipids also emerged as important cell death players in plants. AAL toxin can cause depletion of complex ceramides and accumulation of precursors and the combination of the two events somehow triggers PCD [6]. Not only depletion but also accumulation of ceramides can trigger PCD, as demonstrated by the recent characterization of the first plant ceramide kinase mutant [17]. Thus, a fine balance of sphingolipids is crucial for maintaining cell survival.

With the comprehensive gene expression analysis, we wanted to shed light on the mechanisms by which perturbations in sphingolipid metabolism trigger PCD. At the same time, we aimed at identifying new genes of the cell death machinery and genes that may play a principal role in the regulation and execution of cell death. Changes in mRNA levels do not always translate into similar alterations in protein levels or enzyme activities. Moreover, there may be genes that are not significantly altered in their expression, and yet do have a profound role in PCD. Nevertheless, a comprehensive transcriptome analysis gives an impression about the dynamics of the cellular processes and is an indispensable tool for selecting genes for subsequent reverse genetics studies. Oligonucleotide arrays are becoming the method of choice in terms of high sensitivity and low background noise. Northerns with selected genes were used to verify the array data. The results from the Northerns were consistent with the microarrays.

Distinct stages of the cell death process

We could discriminate distinct stages of the AAL-induced cell death process. At 7 h, a relatively small number of genes were just moderately altered in expression. Nevertheless, this initial stage is very important as many of these genes are key regulatory proteins like transcription factors or components of signalling cascades. Here are the first signs for accumulation of the signalling molecules ethylene and H_2O_2, as judged by elevated transcript levels of ethylene- and ROS-sensitive genes and by in situ DAB staining for the presence of H_2O_2. At 24 h, the number of regulated genes doubled and the alterations in expression were much greater as expressed by the fold change in transcript levels. More important, genes not previously expressed were suddenly switched on to appreciable levels. These genes included proteases and genes related to the oxidative burst as well as a diverse group of genes not previously associated with cell death. In addition, more transcription factors were upregulated. We also see much stronger accumulation of H_2O_2 and also synthesis of callose, which supports the idea of an oxidative burst. On the other hand, no sphingolipid-related genes were significantly altered. At 48 h, we observed a global transcriptional reprogramming, as the number of regulated genes rose more than tenfold. This was the time when most of the auxin-regulated and photosynthesis-related genes were repressed and genes from the proteasome pathway induced. The previously identified proteases were further induced. At the same time, we observed the first signs of nuclear condensation, a morphological change that can occur during plant PCD [10]. At the final stage 3 days after the application of the toxin, cell death occurred, as estimated by increased electrolyte leakage and leaf necrosis. The transcriptome is similar to the previous time point. The stages unfolded the natural progression of PCD and revealed not only the timing of the events but also the genes participating in each stage.

An oxidative burst and production of ethylene are two early events triggered by the AAL toxin and both have important roles in regulating PCD

The most upregulated genes at the first time point were EREBP-3-like and a GST, genes known to respond to eth-

ylene and H_2O_2, respectively [20, 21]. EREBPs are transcription factors induced by ethylene and involved in ethylene responses [20]. A number of transcripts for ROS-generating enzymes were switched on at the second time point: a respiratory burst oxidase AtRbohC, germin oxalate oxidase, and several peroxidases and oxidoreductases with the ability to generate ROS, suggesting a biphasic oxidative burst involvement in the cell death process [22, 23]. The upregulation of these genes persisted and even became stronger at the next two time points, indicating a sustained and more prominent second phase of the oxidative burst. The second phase is the one that triggers PCD during the hypersensitive response [11]. Recently, two other *Arabidopsis* respiratory burst oxidase gp91(phox) homologues, AtrbohD and AtrbohF, were shown to be required for accumulation of ROS in the plant defence response [24]. Germins with oxalate oxidase activity have emerged as main generators of H_2O_2 in cereals during incompatible plant-pathogen interactions [25]. Recently, a wheat oxalate oxidase expressed in sunflower led to accumulation of H_2O_2 and triggering defence responses [26]. However, to date the function of germin-like proteins from dicots remains uncharacterized with regard to their biochemical properties or antimicrobial activities [25]. Support for the biphasic oxidative burst came from the in situ visualization of H_2O_2 accumulation. During the first phase at 7 h, only few single cells accumulated H_2O_2, while in the second phase, many more cells showed greater accumulation of H_2O_2 and this coincided with the elevated transcript levels of more ROS-generating enzymes.

H_2O_2 is a versatile signalling molecule which at low concentrations can induce stress acclimation while at high concentrations can trigger cell death [27, 28]. In *Arabidopsis*, H_2O_2 can also negatively regulate the auxin responses through activation of a MAPK cascade [21]. We see that many auxin-regulated genes are repressed at the third time point, right after the induction of the genes encoding ROS-generating enzymes. These molecular data suggest that the AAL toxin will interfere with growth and development, as auxin is known to be involved in the regulation of these processes. Indeed, our experiments show that the LOH2 mutant cannot grow when seeds are germinated in the presence of as little as 40 nM AAL toxin. The effect is distinct from the light-dependent leaf cell death, as initially seeds germinate but the newly emerged seedlings do not grow and eventually die 2 weeks after germination (data not shown).

ROS can increase ethylene production through activation of 1-aminocyclopropane-1-carboxylic acid synthase (ACC synthase) and transcription upregulation of 1-aminocyclopropane-1-carboxylic acid oxidase (ACC oxidase), two enzymes involved in ethylene biosynthesis [20]. In turn, ethylene can greatly potentiate the oxidative burst [29]. In this way, the two signalling molecules can stimulate each other's production, thus overamplifying the initial signal. In agreement with this, we see late upregulation of an ACC oxidase gene (At1G01480) in addition to the early induction of EREBP-3. These findings establish a principal role of ethylene in the cell death process, as ethylene is also a key regulator in other types of PCD including senescence, a developmental PCD, and campothecin-induced PCD [29, 30]. In our experiments, inhibition of ethylene production led to a substantial decrease in cell death. All these results together suggest that genes regulated by ethylene and ROS are important modulators of plant PCD.

In recent years, NO has emerged as a versatile signalling molecule regulating a myriad of biological processes in plants, including cell death responses [31, 32]. We showed that generation of NO is important for the enhancement of AAL-toxin-induced cell death as plants unable to synthesize NO were significantly compromised in the PCD response. Despite the progress in revealing the role of NO in PCD, there is still debate about the main source of NO in plants. There are at least two potential sources: nitrate reductase and the pathogen-inducible P protein of the glycine decarboxylase complex [31, 33]. In our experiments, we observed induction of one of the genes encoding nitrate reductase, but the significance of this finding remains to be elucidated. We also noticed strong induction of two monooxygenases annotated as NO forming due to their analogy with animals. Whether these are new players in PCD also remains to be evaluated. Although the question as to how NO synthesis is exactly stimulated still stays open, there is plenty of evidence for a complex interplay between ROS and NO. In a balanced model attempting to explain that interplay, Delledone and co-workers propose that ROS and NO act together to determine cell survival or death [34]. Figure 5 summarizes the results presented here and proposes a model of action of the AAL toxin. In addition to ethylene and hydrogen peroxide presented in this model, other signalling molecules like salicylic acid can also contribute to cell death. These signals interplay with each other to determine the ultimate fate of the plant cell.

Transcription factors and the global transcriptional reprogramming.

The complex interplay between ROS, ethylene and NO results in early induction of a diverse group of transcription factors that later orchestrate the global switch in expression. In addition to the three EREBPs, several other transcription factors are induced early on: a zinc finger C2H2 protein, a basic helix-loop-helix protein, two MYB family members, two NAM transcription factors and a WRKY family transcription factor. On the other hand, two CONSTANS B-box zinc finger transcription factors are downregulated. At the second time point, more WRKY and NAM transcription factors are upregulated.

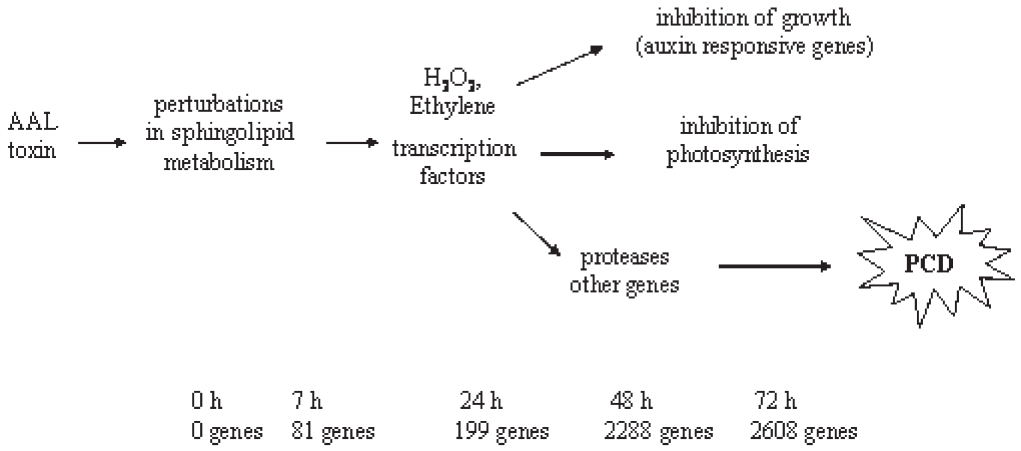

Figure 5. Proposed model of action of AAL toxin. The toxin leads to perturbations in sphingolipid metabolism (depletion of complex ceramides and accumulation of precursors) which in turn leads to generation of secondary signals such as H_2O_2 and ethylene and altered expression of regulatory proteins, for example, a number of transcription factors. The latter leads to transcriptional reprogramming resulting in several distinct responses such as inhibition of growth and photosynthesis and eventually PCD. Below, the four time points (in hours) and the number of regulated genes at these time points are indicated.

WRKYs are a large family of plant-specific transcription factors that bind to the W-box of promoter regions of many pathogenesis-related genes [35]. Indeed, we observed strong induction of several PR genes. However, WRKYs are not confined to PR induction. The members of this family (74 in *Arabidopsis*) have diverse biological functions ranging from stress responses to development [36]. Interestingly, the two developmental processes in which WRKY transcription factors are implicated are trichome development and senescence, and in both cases PCD is indispensable for the normal processes to occur [37, 38].

In contrast with WRKYs, the involvement of DREB-like AP2 domain transcription factor and NAM transcription factors in PCD is not clear. Equally unclear is the link between the CONSTANS family of transcription factors and PCD. The CONSTANS genes in *Arabidopsis* have an important role in the regulation of flowering by photoperiod [39, 40].

A recent analysis of H_2O_2-induced cell death gene expression in tobacco also identified WRKY, NAM and MYB transcription factors as among the earliest genes induced [41]. Vandenabeele and co-workers also reported induction of SCARECROW transcription factors but not upregulation of C2H2-type zinc finger and DREB-like AP2 domain transcription factors nor downregulation of CONSTANS transcription factors. Future work needs to determine the precise role of these transcription factors in the cell death response.

Proteases and AAL-toxin-induced cell death

In animals, proteases play a key role in the regulation of PCD. The two major animal proteolytic systems linked with PCD are the caspases and the proteasome pathway, although other proteases can also be involved in the cell death process [14]. Indeed, proteolytic activity is essential for plant PCD [42, 43]. However, no specific plant proteases have been ascribed a principal role in the cell death process. Moreover, particular proteases are yet to be associated with some types of PCD. For example, cysteine protease activity is important for PCD triggered by hydrogen peroxide in soybean cells, but the precise cysteine proteases remain to be identified [43]. There is also a caspase-like activity in plants, but the caspase-like genes remain elusive [44].

We observed induction of a distinct set of proteases from several different groups. Among them is an aspartic protease with high similarity to nucellin, a protease involved in nucellar degeneration in barley [12], a matrix metalloproteinase and a subtilisin-like protease. At the same time, the senescence-associated protease SAG12 was not induced in our samples, demonstrating that AAL-toxin-induced cell death is distinct from senescence. Serine protease activity is required for apoptotic cell death in tobacco cell suspension cultures [45]. Moreover, plant subtilisin-like serine proteases may initiate signal transduction pathways by processing bioactive cell wall peptides [41].

In recent years, different components of the plant proteasome pathway have been implicated in processes requiring PCD, including catalytic or regulatory proteasomal subunits and components of the large family of E3 ubiquitin-protein ligases [10, 46, 47]. In our experiments, we observed upregulation of many components of the proteasome pathway, including the ubiquitin-specific protease UBP14 and genes from the regulatory and catalytic subunits of the proteasome. More important, a C3HC4-type zinc finger (RING) was highly induced and the in-

duction actually preceded that of the other genes from the proteasome pathway. RING fingers have E3 activity and the E3 enzymes are the determinants of the specificity for proteins subjected to proteasomal degradation. Substantiating these findings, a recent study on genes induced during H_2O_2-dependent cell death also found many members of the proteasome pathway upregulated, including regulatory and catalytic subunits as well as E2 and E3 enzymes [41]. The key to understanding the exact place of the plant proteasome in the cell death network will be identification of the potential proteasomal targets.

New players in PCD?

While a role of the transcription factors and proteases in PCD is easily envisaged, little is known about oxoglutarate-dependent dioxygenases, FAD-linked oxidoreductases and some of the other strongly regulated genes. Most of these highly regulated genes were not previously connected with PCD. Although it is not clear whether they are really related to PCD or are merely a consequence of the global changes occurring during PCD, their relatively early regulation suggests the former. This group is heterogeneous and includes proteins with diverse functions ranging from transport to hydrolysis and oxygenation. The transport proteins are represented by several ABC transporters, one of the biggest plant families, a heavy metal transport protein and a putative lipid transfer protein. Other groups represented in table 1 are cytochrome p450 family members and several biotic-stress-related proteins. The latter are particularly interesting as they reveal a connection between biotic stress and PCD. The most regulated gene among this group is At2G35980.1, similar to harpin-induced protein hin1 from tobacco. The *Arabidopsis* genome contains a family of NDR1/HIN1-like (NHL) genes that show homology to the non-race-specific disease resistance (NDR1) and the tobacco (*Nicotiana tabacum*) harpin-induced (HIN1) genes. Recently, overexpression of NHL3, a pathogen-responsive member of this NHL gene family, correlated with increased resistance to *Pseudomonas syringae pv. tomato* [48].

Another distinctly upregulated gene is GAD1. It encodes glutamate decarboxylase, a Ca^{2+}/calmodulin-dependent enzyme that converts glutamate to γ-aminobutiric acid (GABA) [49]. GABA is regarded as a stress molecule and participates in the so-called GABA shunt, a pathway that is essential for restriction of levels of ROS in plants [50].

We were surprised to see levels of a number of drought- and low-temperature-induced proteins, including LTI78 and the transcription factor DREB1C, going down already at the first time point. LTI, also known as COR78/RD29A, protects plants under freeze-induced dehydration and is induced under high light, drought, cold and salt stress [51]. This suggests that lowering the plant protective mechanisms may contribute to the cell death.

A substantial number of the most regulated genes at the first two time points are annotated as unknown or expressed proteins. As new annotations and new functional data emerge regularly, we will soon know more about these genes and try to find their place in the plant cell machinery.

Further functional studies are needed to determine the precise role of the identified proteases, transcription factors and the other transcriptionally altered genes in the regulation and execution of the cell death process.

Acknowledgements. We thank S. D. Spassieva for the AAL toxin, J. E. Markham for the LOH2 plants, and B. Venema for greenhouse assistance. We are also grateful to W. van Workum for helpful discussions regarding the experiments with ethylene. I. Gadjev acknowledges Nuffic for a Huygens fellowship. ABRC is acknowledged for the DNA stocks used for the Northerns.

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